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Piperine Decreases Binding of Drugs to Human Plasma and Increases Uptake by Brain Microvascular Endothelial Cells

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Abstract

We previously reported that piperine, an active alkaloidal principal of black and long peppers, enhances drug bioavailability by inhibiting drug metabolism. Another mechanism influencing drug availability/uptake is its free fraction. Since piperine is highly lipophilic, we hypothesize that it could also interact with drugs through binding displacement and influence their bioavailability. Accordingly, using equilibrium dialysis, we investigated whether piperine alters the binding of model drug ligands i.e. flunitrazepam, diazepam, warfarin, salicylic acid, propranolol, lidocaine and disopyramide to human plasma (n=4). Since, alterations in binding influences drug disposition, we also studied the effects of piperine on the uptake of plasma bound ^{3}H-propranolol and ^{14}C-warfarin by cultured bovine brain microvascular endothelial cells (BMECs). Piperine (1-1000μM) increased the free fraction (fu) of both albumin and alpha-acid glycoprotein (alpha-AGP) bound drugs in a concentration-dependent manner ($P<0.01$). Moreover, piperine (10μM) increased the uptake of ^{3}H-propranolol and ^{14}C-warfarin by BMECs ($P<.01$). In conclusion, our findings provide the first evidence that piperine displaces plasma bound drugs from both albumin and alpha-AGP, and facilitates drug uptake across biological membranes (e.g. BMEC). Moreover, it is feasible, that piperine may similarly facilitate the transport of drugs into tissues, in vivo, and alter both pharmacokinetics and pharmacodynamics of administered drugs.

Introduction:

Drug action in the body depends on the rate of metabolism, blood flow, and extent of binding to plasma proteins (Dayton et al., 1973; Wilkinson and Shand, 1975; Wilkinson, 1987; Rowland and Tozer, 1980). Alterations in either characteristics can potentially effect the body distribution of the compound as well as its removal from the body, thereby modulating its pharmacological action (Dayton et al., 1973; Wilkinson and Shand, 1975; Wilkinson, 1987; Rowland and Tozer, 1980). Moreover, dietary molecules can influence the pharmacokinetics and pharmacodynamics of drugs by altering drug binding as well as metabolism (Melander, 1978). Piperine (1-piperoyl piperidine) a major active ingredient of black and long peppers **(Figure 1),** which is widely used in foods and traditional systems of medicine has been shown to enhance bioavailability of drugs (Srinivasan 2007). For example, piperine increases the plasma concentrations of test drugs such as fexofenadine (Bedada and Boga, 2017; Jin and Han 2010), phenytoin (Bano et al., 1987), propranolol (Bano et al., 1991), theophylline (Bano et al., 1991), rifampicin (Zutshi et al., 1985), in humans. Earlier we suggested an explanation to this phenomenon based on the compound's strong inhibitory effect on hepatic and intestinal drug metabolising enzymes (Atal et al., 1985; Reen et al., 1996) both in-vivo and in-vitro and offered it as the plausible explanation for the mechanism by which it could enhance drug bioavailability and hence prolong drug action. Although inhibition of the monoxygenases by certain compounds can enhance bioavailability of drugs (Sontaniemi, 1993) and alter their pharmacokinetics and pharmacodynamics (Dayton et al., 1973; Wilkinson and Shand, 1975; Wilkinson, 1987; Rowland and Tozer, 1980), other mechanisms such as alterations in plasma binding may also be involved.

Drug molecules circulate in blood in two rapidly interchangeable forms (Daytton et al., 1973) bound to plasma proteins such as albumin, and/or α-acid glycoprotein (Wilkinson and Shand, 1975) free or unbound form (Kwong, 1985). The extent of drug binding to plasma proteins has important implications on drug disposition and drug action (Dayton et al., 1973; Wilkinson and Shand, 1975; Wilkinson, 1987; MacKichan, 1989). Drug effect often depends on the free drug in plasma, because it is this fraction of drug which can cross capillary and cell membranes and hence is an important determinant for its ability to leave the vasculature and gain access to receptors, tissues, various sites of elimination and metabolism (MacKichan, 1989). Alterations in plasma protein binding is known to effect drug disposition (Wilkinson and Shand, 1975; Wilkinson 1987; Martin, 1965; Jusko and Gretch, 1976). Change in the free fraction alters hepatic elimination and distribution (Wilkinson and Shand, 1975; Wilkinson, 1987) with resulting changes in concentration at the site of action. Piperine, being highly lipophilic, could potentially effect the binding interaction of drugs to plasma proteins and hence, influence the disposition parameters of plasma bound drugs. Accordingly, we investigated the effects of piperine on the binding interaction of a number of acidic and basic drugs to plasma from healthy subjects. Additionally, we investigated the effects of piperine on the uptake of propranolol and warfarin by cultured bovine brain microvascular endothelial cells.

MATERIALS AND METHODS

Materials:

Diazepam, flunitrazepam, warfarin, salicylic acid, propranolol, lidocaine, antipyrine, disopyramide, phenazine and piperine were purchased from Sigma Chemical Co. (St. Louis, MO). Biodegradable scintillation fluid, acetonitrile, methyl-^{3}H-diazepam (57 mCi/mmol), N-methyl-^{3}H-flunitrazepam (57 mCi/mmol), 1-^{14}C-warfarin (46 mCi/mmol), DL-^{3}H-propranolol (18 mCi/mmol), carbonyl-^{14}C-lidocaine hydrochloride (48 mCi/mmol), were obtained from Amersham Corp. (Arlington Heights, Ill.); 7-^{14}C-salicylic acid (58 mCi/mmol) was from Du Pont Co. (New England Nuclear Research Products, Boston, Mass.); N-methyl-^{14}C-antipyrine (50 mCi/mmol) from ICN Radiochemicals (Irvine, CA.); and 3-^{14}C-disopyramide (4.3 mCi/mmol) from G.D. Searle and Co. (Chicago, Ill). All radiolabelled drugs were >98% pure as determined by thin layer chromatography. Dialysis microcells and semipermeable membrane (mol. wt. cut off 12,000 to 14,000 kd; Dianorm dialyzer with spectrapor membrane No.2) were procured from Spectrum Medical Industries (Los Angeles, CA.) and Ultrasphere column (5-μm, octadecyl 25 x 4.6mm) from Beckman Instruments Inc. (Fullerton, CA). Tissue culture: dispase, deoxyribonuclease-I, rat tail collagen, fibronectin, endothelial cell growth supplement, penicillin-G, streptomycin, polymyxin-B, amphotericin-B, heparin, tris-base, were purchased from Sigma; collagenase/dispase from Boehringer-Mannheim biochemicals (Indianapolis, Ind.); Minimum essential medium (MEM), DMEM-F12 Ham nutrient mix HEPES, from Gibco laboratories (Grand Island, NY); fetal calf serum from Hyclone Laboratories (Logan, UT); plycarbonate membranes (13mm diameter, 12-μm pore size) from Nucleopore Co., (Pleasanton, Calif.); Percoll from Pharmacia LKB

Biotechnology Inc. (Piscataway, NJ); and immunohistology kit for endothelial cell characterization (Rabbit anti-factor VIII polyclonal antibodies) from Biogenex Labs. (Dublin, CA). All other tissue culture and drug binding chemicals used were of the best available grades.

Drug Binding Studies:

Blood (50ml) was collected by venepuncture into EDTA-treated tubes from healthy male subjects/volunteers (n=4) with an average age of 30±2 years and following the institutional ethical guidelines at that time. The subjects were fasted overnight and had taken no medications for at least 1 week prior to obtaining the blood sample, and were non-smokers. Plasma was obtained by centrifuging blood at 1000g for 30 min at 4°C. Drug standards were added to the freshly aspirated plasma in the absence or presence of piperine (1, 10, 100 or 1000 µM) to achieve a concentrations typical of those observed in clinical studies and as follows: diazepam, 0.1µg/ml; flunitrazepam, 0.1 µg/ml; warfarin, 1µg/ml; salicylic acid, 1µg/ml; propranolol, 0.1µg/ml; lidocaine, 3µg/ml; antipyrine, 10µg/ml and disopyramide, 10µg/ml. Each of the standard solutions contained a tracer quantity of radiolabelled drug appropriately diluted with unlabelled compound to produce the required total concentration.

Plasma binding was determined by equilibrium dialysis at 37°C using microcells and a semipermeable membrane (Mol. wt. cut-off 12,000-14,000 kd). Plasma (0.2ml) was dialysed against an equal volume of 0.067 mol/L phosphate buffer, pH 7.4 for 4 hours. The percentage of drug present in plasma in unbound form calculated using following equation:

$$\% \text{ Unbound drug} = \frac{\text{Drug concentration in buffer}}{\text{Drug concentration in plasma}} \times 100$$

Drug concentrations of the radiolabelled ligands was determined by liquid scintillation counting with external standardization of 50 µl of plasma or buffer added to 15 ml counting scintillation fluid.

Isolation and Culture of Brain Microvascular Endothelial Cells:

Microvascular endothelial cells were isolated from bovine brain according to the method of Audus and Borchardt (Audus and Borchardt, 1987), with minor modifications. Briefly, the cortical tissue of two or three bovine brains obtained from the local abattoir was cleaned of mennings and all large vessels. The gray matter from the cortical region was dissected, minced, and passed through a sterile stainless steel wire mesh (1mm square), and digested for 20-30 min at 47°C with a solution of 12.5% dispase (4ml /50gm tissue) and deoxyribonuclease-I (20µg/ml) in MEM buffered with 50µM HEPES. Subsequent to this incubation, Dulbeccos' minimal essential medium (MEM) (equal to the weight of grey matter) buffered with TRIS-base (pH 9.5) was added and the mixture further incubated for 2.5-3 hours. After digestion with dispase the cell suspension was centrifuged at 3200 rpm for 10 min and the pellet suspended in 500 ml of dextran (13%) and centrifuged at 7750 rpm for 10 min. The crude capillaries were obtained as a pellet whereas, the floating cell debris, fat, myelin was discarded. Capillary pellet was further

digested for 4hrs at 37°C with a solution of collagenase/dispase (1mg/ml) and deoxyribonuclease-I (20µg/ml). The digested suspension was centrifuged at 1000g for 10 min, and the pellet suspended in 8ml MEM and 2ml aliquots of it layered over 50ml of pre-established percoll gradient and centrifuged at 3750 rpm for 10 min. Endothelial cells were collected from the percoll and washed and suspended in complete culture medium (1:1 mixture of MEM and DMEM-F12 ham, 10mM HEPES, 13mM $NaHCO_3$, 100 µg/ml penicillin G, 100 µg/ml streptomycin, 50µg/ml polymyxin B, 2.5µg/ml amphotericin B, 100 µg/ml heparin, 25µg/ml endothelial cell growth supplement and 20% fetal calf serum). Cell viability was determined by trypan blue exclusion technique and isolated cells for later use were frozen at -70°C in complete culture medium supplemented with 10% dimethyl sulfoxide.

Frozen or freshly obtained endothelial cells were thawed, suspended in complete culture medium and plated at a density of 2.5 x 10^6 cells/100mm in a culture dish containing polycarbonate membranes (13mm diameter). The polycarbonate filters had been fixed to the culture dish surface and coated with rat-tail collagen and fibronectin (40 µg/ml), cross linked with ammonia fumes, and sterilised under UV light as described before (Audus and Borchardt, 1987). The medium was changed every day after four days of plating and the cells reached confluence in 10-12 days as determined visually under a light microscope. The purity of the cells was established morphologically and by immunofluorescent staining the cells to test for the presence of anti-Von willebrand factor VIII as described before (Audus and Borchardt, 1987) using monoclonal antibodies.

Drug Uptake Studies:

Brain microvascular endothelial cells (BMECs) play a key role in regulating transport of drugs across the blood-brain barrier. Cultured BMECs have been widely used as a model to assess the transport of drugs across BBB, in vitro, as well as uptake of drugs (Audus and Borchardt, 1987). Since free fraction of drugs is postulated to cross BBB (Dubey et al., 1989) we used BMECs to assess the effects of piperine on drug uptake. Endothelial cells grown to confluence on polycarbonate filters were incubated with plasma containing 0.1µg/ml propranolol or 1.0µg/ml warfarin (close to clinical concentrations), and pulsed with 10.5 x 10^6 dpm/ml of ^{3}H-propranolol or 15 x 10^6 dpm/ml ^{14}C-warfarin, in the absence or presence of 10 µM, piperine, at 37°C for 5, 15, 30, 60, and 120 seconds. Immediately after incubation the filters were washed 4 times by carefully suspending them in ice-cold phosphate buffered saline. The filters were placed in scintillation vials and treated overnight with 0.5ml of 0.3N NaOH in 1% sodiumdodecyl sulphate and drug concentrations determined by liquid scintillation counting. In separate experiments conducted in parallel, the effect of piperine on cell viability was determined by looking for trypan blue exclusion. Moreover, the loss of cells during treatment were assessed by counting the cells after trypsinization and by looking at monolayers under a light microscope after treatment.

Statistical Analysis: Differences in the plasma binding of drugs in piperine treated and untreated groups were evaluated by the paired Student's t-test, whereas the differences in drug uptake and cell viability in treated and untreated groups was evaluated by ANOVA, utilising intergroup comparisons by Fisher´s Least Significant Difference Test (Numbers Cruncher Statistical System, Kaysville, UT). In both cases $p<0.05$ was taken as the minimum level of statistical significance.

RESULTS

Effect of Piperine on the Binding of Drugs to Human Plasma:

Piperine (**Figure 1**) being highly lipophilic in nature bound extensively to human plasma, with a free fraction of 0.015 ± 0.004. Pre-treatment of plasma with different concentrations of piperine significantly ($p<0.01$) altered the binding of both acidic and basic drugs in a concentration dependent manner (**Figure 2A and 2B**). In presence of piperine (100 µM) the increase in free fraction of various acidic drugs (mean ± S.D., n=4) was: 2.74±0.06 fold ($p<0.01$) for flunitrazepam; 3±0.16 fold ($p<0.01$) for diazepam; 4.6 ±0.08 ($p<0.01$) warfarin; 2.4±0.09 ($p<0.01$) salicylic acid, and for basic drugs was: 1.9± 0.03 fold for propranolol ($p<0.01$); 1.5±0.02 fold ($p<0.01$) for lidocaine; 1.5±0.04 fold for disopyramide ($p<0.01$).

There was no effect of piperine on the binding of antipyrine at any concentration. Since 100 µM piperine significantly increased the unbound fraction of all drugs, except antipyrine, we chose this concentration of piperine to assess the effects on bound to free ratio. The relative decrease in bound to free ratios of these drugs at 100 µM piperine concentration was in the order: warfarin > diazepam > flunitrazepam > salicylic acid > propranolol > lidocaine > disopyramide (**Figure 3**). In presence of 100 µM piperine the bound to free ratio (B/F) of albumin bound drugs (diazepam, flunitrazepam, warfarin, salicylic acid) decreased by 3.16±0.8 fold, whereas, the bound to free ratio of alpha-AGP bound drugs (propranolol, lidocaine, disopyramide) decreased by 1.65±0.05 fold (**Figure 3**). The decrease in B/F ratio of albumin bound drugs was significantly higher ($p<0.01$) than those bound to α-AGP.

Effect of Piperine on Cell Viability:

Since the uptake studies were conducted for 2 min, we chose a longer i.e. 15 minute period to study if the effects of piperine on drug uptake were not compromised by cell toxicity. Treatment with the highest concentration (1mM) of piperine for 15 min did not induce cell toxicity. The BMEC viability (trypan blue exclusion) in monolayers treated with vehicle or 1 mM piperne for 15 min was 99 ± 2 % and 98.5 ± 3 %, respectively (P > 0.05 vs vehicle). No floating dead cells were detected in the medium of BMEC monolayers treated for 15 min with vehicle and piperine 1mM, respectively. Moreover, the dead cell count in monolayers grown on polycarbonate filters treated with or without piperine did not differ.

Effect of Piperine on Uptake of Drugs:

Piperine significantly increased the free fraction of warfarin and propranolol at a concentration of 10µM. Since free fraction of drug governs its uptake, we selected this concentration for the uptake studies using monolayers of BMECs **(Figure 4A).** Uptake of propranolol and warfarin by BMECs was time dependent. In absence of piperine warfarin uptake by BMECs was significantly lower than that observed for propranolol ($p<0.05$). However, in the presence of 10 µM piperine the uptake of warfarin and propranolol by BMECs was significantly increased ($p<0.01$; **Figure 4B and 4C**, respectively). The increase in uptake after 60 seconds of incubation was 3.8±0.4 fold and 2.1±0.04 fold, respectively. This increase in uptake was proportional to the increase in free fraction of propranolol and warfarin which in the presence of 10 µM piperine increased, from 0.229±0.05 to 0.411±0.02 for propranolol ($P<.05$) and from 0.009±0.003 to 0.022±0.002 for warfarin ($P<.05$).

DISCUSSION

Drug action in the body depends on the rate of absorption, distribution, metabolism and elimination (Dayton et al., 1973; Wilkinson and Shand, 1975; Wilkinson, 1987; Rowland and Tozer, 1980). The pharmacological response obtained is for a majority of drugs, related to the concentration of drug at receptor site(s) (MacKichan, 1989). Moreover, the binding of blood-borne endogenous or exogenous ligands to plasma constituents is an important determinant of the compounds distribution in and removal from the body (Wilkinson, 1987). The binding affinity of drugs to plasma proteins defines their ability to diffuse across the vasculature and gain access to receptors in various organs or tissues. Moreover, it governs the elimination and metabolism characteristic of a drug. Indeed, changes in a compound's binding to plasma proteins can result in altered drug distribution and unexpected pharmacological responses (Dayton et al., 1973; Svensson et al., 1986). Plasma albumin is quantitatively the most important protein to which a large number of drugs, particularly the neutral and acidic (anionic) drugs, bind in a reversible fashion, (Sjoholm et al., 1979). Albumin contains a number of sites with differing specificities. Warfarin for example, binds with high affinity to site-I, which is also the primary binding site for bilirubin, whereas benzodiazepines and tryptophan interact more specifically with site-II. By contrast, the albumin binding of salicylic acid involves both these sites. While acidic drugs bind exclusively to albumin, drugs with basic character bind to α-acid glycoprotein more extensively, than to plasma albumin (Kwong, 1985, MacKain, 1989; Matin, 1965). Moreover, as compared to albumin, alpha-AGP contains only one binding site to which basic and some typically site-I albumin binding drugs may gain access to.

Drug binding, can be modified by the presence of other exogenous or endogenous compounds, which can compete for the same binding sites (drug displacement by competitive inhibition). Alternatively, two ligands can be bound to two separate sites and mutually effect each other's binding by free energy coupling between the two sites (Weber, 1975). Our observation that piperine increases the free fraction of drugs which bind to albumin (diazepam, flunitrazepam, warfarin, salicylic acid) and alpha-AGP (propranolol, lidocaine, and disopyramide), strongly suggests that piperine binds to both albumin and alpha-AGP. Comparison of the displacing effects of piperine on the binding of both acidic and basic drugs to human plasma demonstrates that the effect is more pronounced for acidic drugs. Moreover, it suggests that piperine is more effective in displacing albumin bound drugs. A two fold difference in the bound to free ratio of warfarin (site-I) and salicylic acid (site-II) suggests a higher affinity of piperine for site-I. Together, these findings suggest that piperine effectively displaces highly plasma bound drugs from both site-I and site-II of albumin and alpha-AGP.

For drugs that bind to more than one protein in plasma, significant displacement from one of the binding proteins may not result in a demonstrable change in unbound fraction and result in unaltered pharmacokinetics and pharmacodynamics. However, the observation that piperine displaces drugs, which are bound to both albumin and alpha-AGP (disopyramide, propranolol, and lidocaine) suggests that piperine may also be effective in altering the pharmacokinetics and pharmacodynamics of these drugs.

Distribution of drugs from the blood into the tissues occurs by the passage of drugs with low molecular weights (<1000) through the layer of endothelial cells of the capillaries, and binding of drugs to plasma proteins restricts the extravascular distribution of drugs (MacKichan, 1989). The observation that the uptake of propranolol and warfarin by endothelial cells was significantly increased in presence of piperine, and that there was a parallel increase in the free fraction of these drugs, suggests that the enhanced uptake of warfarin and propranolol was related to increase in free fraction of the drugs in presence of piperine. It is plausible that similar effects could occur in-vivo and result in enhanced transport, of drugs (displaced by piperine) across the biological membrane and into the tissues. Accordingly, the displacement of alpha-AGP or albumin bound drugs by piperine might result in altered pharmacokinetics and pharmacodynamics.

Since, plasma binding is also an important determinant of the systemic clearance of a drug, with different effects depending on the free intrinsic clearance of the compound (Dayton et al., 1973; Wilkinson and Shand, 1975; Wilkinson, 1987; MacKichan, 1989). It is conceivable that displacement of drugs by piperine may also result in altered systemic clearance and therapeutic efficacy of the drug. For a poorly eliminated drug, clearance is "restricted" to the unbound moiety and an increase in this fraction results in a proportional enhancement in the clearance of total drug. Although, displacement of drugs by piperine will not affect elimination half-life of displaced drugs with moderate to large volumes of distribution, however, it may shorten the half-life of drugs with small volume of distribution. Since piperine also inhibits hepatic and intestinal drug metabolizing enzymes (Atal et al., 1985; Reen et al., 1996), it is possible that the intrinsic clearance of the drugs will decrease, and this in combination with increased free concentration of the drug may

result in sustained increase in free concentrations of displaced drugs at the site of action. Moreover, piperine induced displacement of drugs (propranolol, lidocaine) whose clearance is not limited by binding to plasma proteins "non-restrictively cleared" could result in immediate and sustained increase in unbound concentration. This may be of clinical significance for drugs with narrow therapeutic indices and might minimally prolong the half-life of drugs with a small volume of distribution, and greatly for drugs with large volume of distribution.

The impact of alterations in protein binding on drug clearance depends on the characteristics of the drug (Dayton et al., 1973; Wilkinson and Shand, 1975; Wilkinson, 1987; Rowland and Tozer, 1980). Clearance of restrictively cleared drugs is limited to free fraction of the drug and clearance of highly bound and restrictively cleared drugs are sensitive to alterations in protein binding. Hence, displacement of restrictively cleared drugs (warfarin) by piperine may result in higher free fraction, which will lead to higher clearance rates, shorter elimination half-lives and a larger fluctuation in peak and trough levels. Furthermore, a transient increase in free drug can be of sufficient magnitude to precipitate an adverse drug reaction if the drug has small volume of distribution and a narrow therapeutic index. However, in case of non-restrictively cleared drugs (propranolol, lidocaine), which are extracted by active mechanisms in both free and bound forms by the liver or the kidney, the clearance is dependent on organ blood flow and relatively independent of protein binding. Hence, alterations in free concentrations of these drugs will not be of clinical significance.

In-vivo the efficacy of the drug not only depends on protein binding, but also greatly influenced by its metabolism. Small concentrations of several drugs reach the circulation when given orally, and are lost due metabolism "first pass effect". Piperine enhances the bioavailability of theophylline, propranolol and phenytoin in healthy volunteers (Melander, 1978; Bano et al., 1987; Bano et al., 1991) and increase the effects of hexobarbital and zoxazolamine in a reversible fashion (Atal et al., 1985). Furthermore, we previously demonstrated that the enhancement of drug bioavailability by piperine was possibly due to reversible and non-competitive inhibition of NADPH-dependent cytochrome P-450-mediated monooxygenases (Atal et al., 1985). In addition, we observed that piperine also lowers the rate glucuronidation in intestine and liver (Singh et al., 1986). Since piperine inhibits both monooxygenases and conjugases in the intestine and liver, sites where the drugs are lost due to first pass effect, it is plausible that higher concentrations of the drug will reach the circulation. However, upon reaching the circulation, the drug is bound to proteins like albumin and alpha-AGP and the delivery of the drug to the target tissue and receptor sites is restricted to the unbound fraction of the drug. Since, piperine displaces drugs from both albumin and alpha-AGP this would result in higher free concentrations of the drug and higher amounts of drug reaching the target tissues and receptors or even increased elimination or clearance of the administered drug. Hence, piperine could potentially alter the pharmacokinetics and pharmacodynamics of drugs irrespective of the route of administration.

Although, our findings provide evidence that piperine reduces drug binding to plasma proteins and increases their uptake by BMECs in vitro, it has limitations. First, whether piperine influences drug binding in vivo and whether this is associated with

enhanced drug bioavailability is unknown. Second, piperine binds reversibly to serum albumin by hydrophobic interactions with a binding constant of 104Mol/L and a stoichiometry of 1:1 preferably at the binding site of subdomain I-B. Even though binding constant of piperine with serum albumin is moderate, major portion of the piperine in blood circulation may still be in the bound form because of very high physiological concentration of albumin (0.6 mM). Based on these characteristics, whether piperine can compete with other drugs for the binding site on serum albumins and consequently make the drugs available in the un-bound form remains unknown and can only be speculated. Third, the serum levels of piperine following oral or intraperitoneal administration are 0.15% of the dose administered, moreover significant presence of piperine metabolite in serum have been reported (Srinivasan 2007; Suresh and Srinivasan 2010; Bajad et al., 2003). Whether circulating concentrations of piperine and its metabolite can influence drug binding in vivo remains unknown. Finally, the fact that piperine increases the uptake in BMECs suggests that it may facilitate transport of drugs across the blood-brain barrier. However, in vivo brain-uptake index studies are required to confirm this possibility.

In conclusion, piperine significantly decreases the binding of both acidic and basic drugs to human plasma and increases the uptake of warfarin and propranolol by BMECs. The findings that piperine inhibits drug metabolism as well as increases the free fraction of plasma bound drugs, suggests that piperine can potentially alter the pharmacokinetics and pharmacodynamics of drugs irrespective of the route of administration. Moreover, piperine may be useful in modulating the effects of clinically used drugs and this possibility needs further investigation.

Abbreviations: Brain microvascular endothelial cells, BMECs; alpha-acd glycoprotein, alpha-AGP; free fraction, fu; Dulbeccos' minimum essential medium, MEM

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Figure Legends:

Figure 1: Chemical structure of piperine.

Figure 2: Bar graph depicting the concentration-dependent effects of piperine (1, 10, 100, 1000 µM) on the binding of: (A) salicylic acid, diazepam, warfarin and lidocaine; and (B) propranolol, flunitrazepam, disopyramide and antipyrine, to human plasma. Results (mean±S.D.) express changes in the unbound free fraction (fu) of the drug in presence or absence of piperine (n=4). * represents P<0.05 versus samples treated with vehicle alone.

Figure 3: Bar graph comparing the effects of piperine (100 µM) on the bound/free ratio of (A) alpha-AGP bound drugs (propranolol [PRL], lidocaine [LDC], disopyramide [DPY]) and (B) albumin bound drugs (diazepam [DZP], flunitrazepam [FNZ], warfarin [WRF], salicylic acid [SA], to human plasma. Results are expresssed as mean±S.D.(n=4). * represents P<0.05 versus samples treated with vehicle alone.

Figure 4: Panel A depicts a representative photomicrograph of a confluent monolayer of brain microvascular endothelial cells used for drug uptake studies. **Panel B** depicts a line graph showing the effects of piperine (10 µM) on the time-dependent uptake of plasma bound ^{14}C-warfarin by bovine brain microvascular endothelial cells. Values at each point represents mean±S.D. (n=4) and expressed as disintegration per minute (dpm)/17.7x10^4 endothelial cells. * represents P<0.05 versus samples treated with vehicle alone. **Panel C depicts** a line graph showing the effects of piperine (10 µM) on the time-dependent

uptake of plasma bound ^{3}H-propranolol by bovine brain microvascular endothelial cells. Values at each point represents mean ± S.D. (n=4) and expressed as disintegration per minute (dpm)/17.7x10^{4} endothelial cells. * represents $P<0.05$ versus samples treated with vehicle alone.

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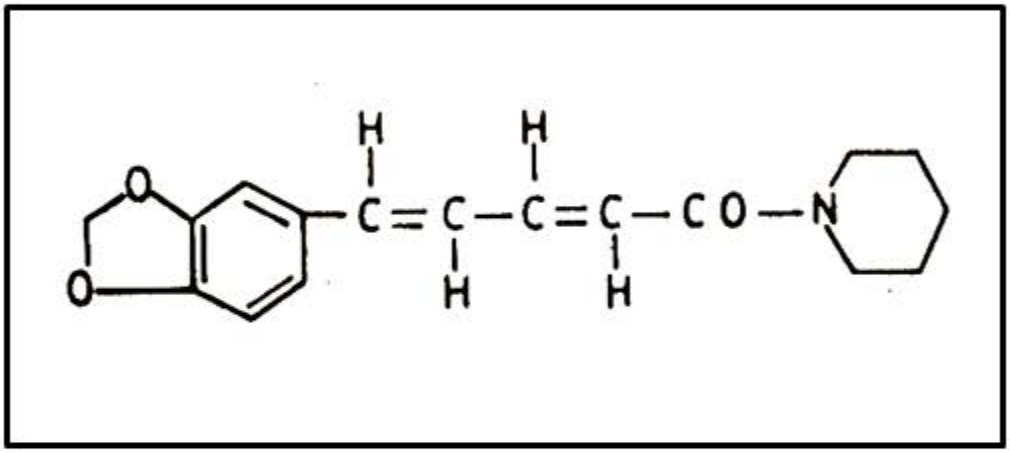
Figure 1



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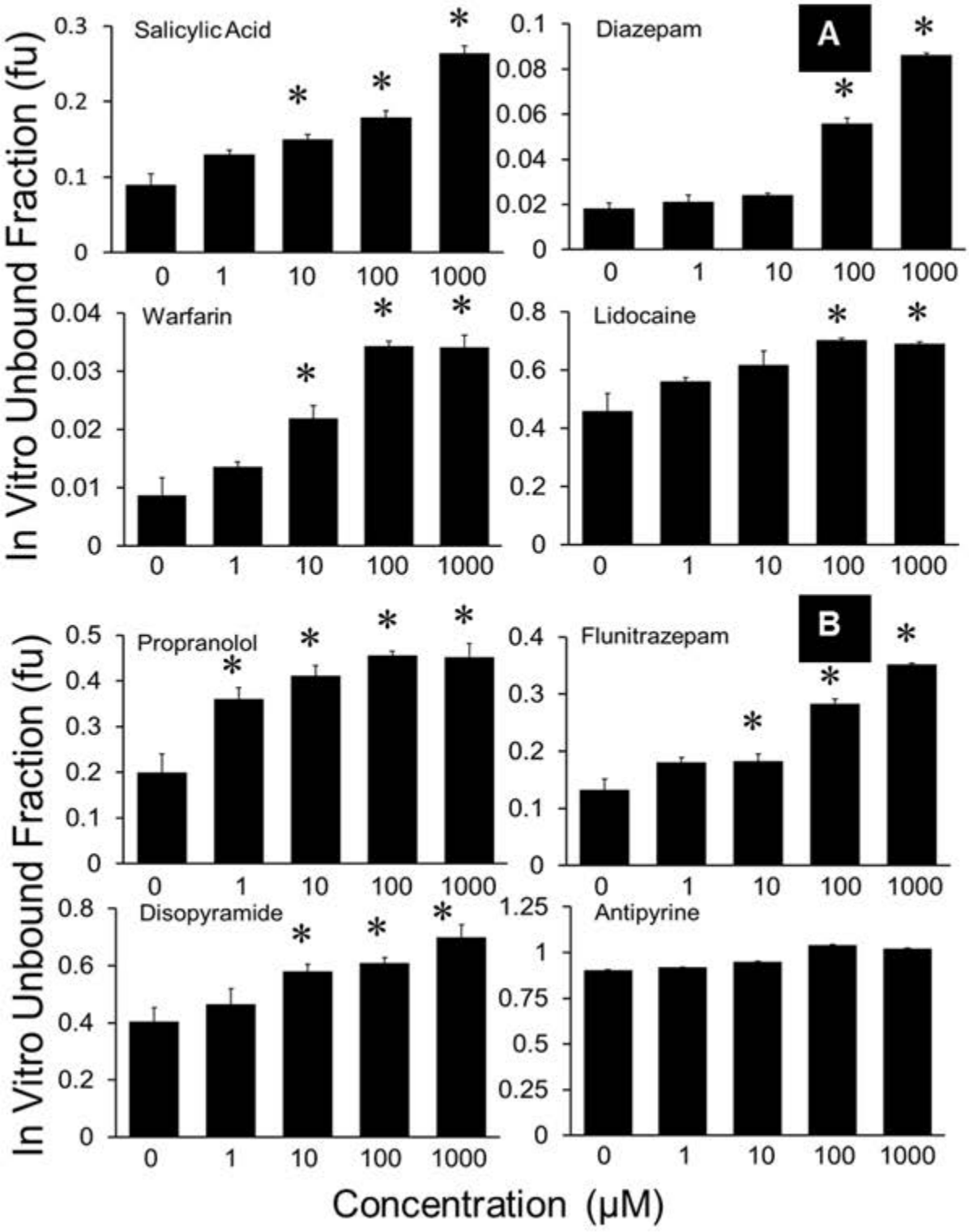

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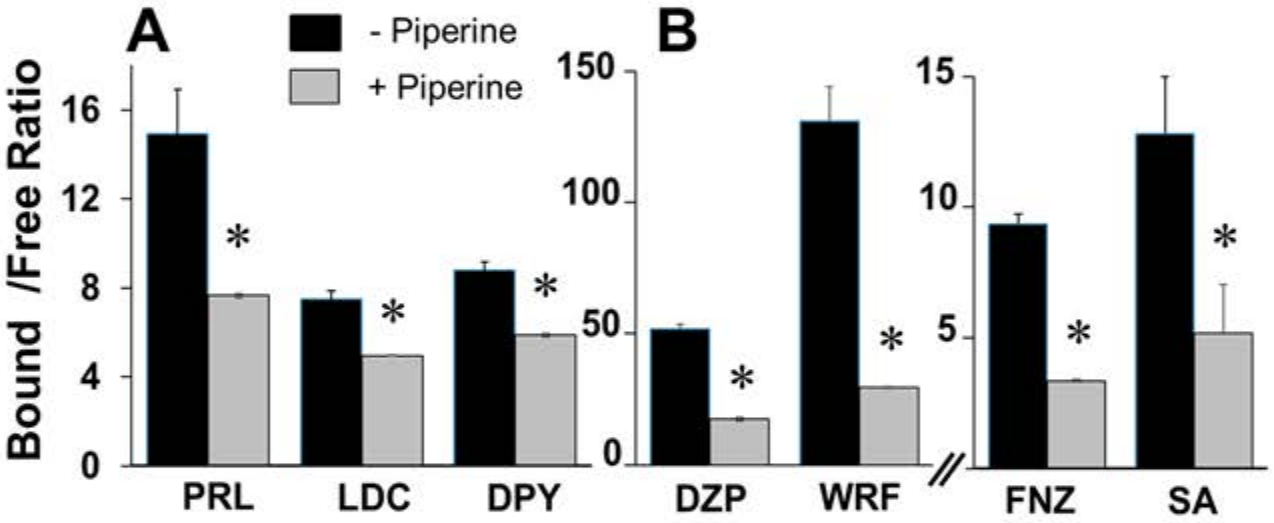
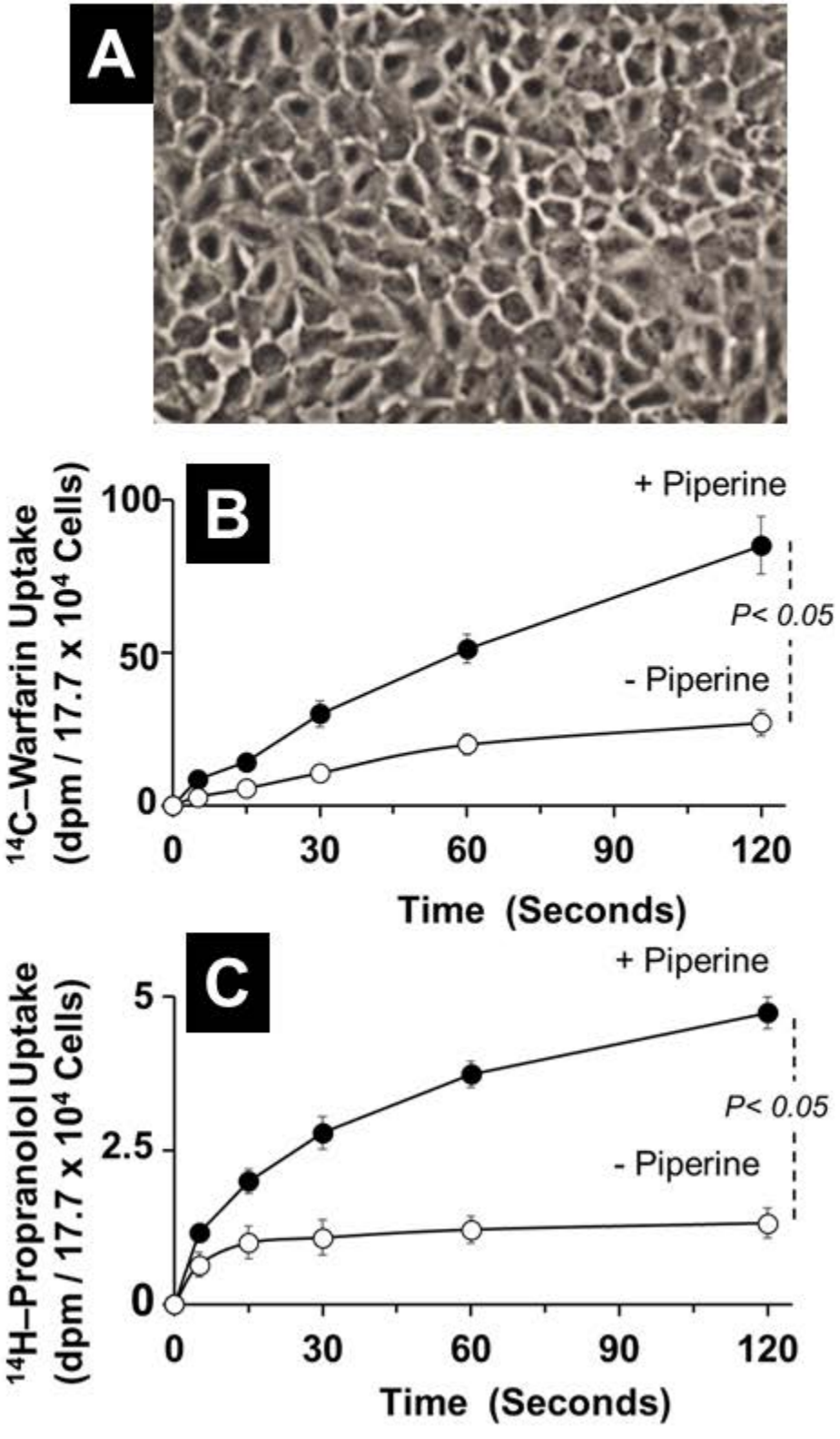
Figure 3




A
B
+ Piperine
- Piperine
P< 0.05
^{14}C–Warfarin Uptake
(dpm / 17.7 x 10^4 Cells)
100
50
0
0
30
60
90
120
Time (Seconds)
C
+ Piperine
- Piperine
P< 0.05
^{14}H–Propranolol Uptake
(dpm / 17.7 x 10^4 Cells)
5
2.5
0
0
30
60
90
120
Time (Seconds)